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(54) Immobilized nucleic acid probe and solid support for nucleic acids.

(57) A solid support capable of binding a nucleic acid thereto upon suitable irradiation, comprising (a) a solid substrate, (b) a photochemically reactive intercalator compound or other nucleic acid-binding ligands, and (c) divalent radical chemically linking the substrate and the ligand (b). Specifically, a hydroxy group-containing solid substrate such as nitrocellulose paper is linked via a bifunctional reagent such as cyanogen bromide or 1,4-butanediol-diglycidyl ether to an amino-substituted angelicin or psoralen or ethidium bromide which in turn is photochemically linked to a nucleic acid. The resulting immobilized nucleic acid probe is capable of hybridizing with complementary nucleic acid fragments and is thereby useful in diagnostic assays.

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PATENT - 0430523

IMMOBILIZED NUCLEIC ACID PROBE AND SOLID SUPPORT FOR
NUCLEIC ACIDS

5 The present invention relates to a novel way of joining a nucleic acid to a solid substrate so as to provide a solid probe suitable for use in various tests, particularly hybridization assays for the determination of specific polynucleotide sequences.

10 In DNA-DNA hybridization and DNA-RNA hybridization assays, one of the complementary nucleic acid chains is commonly coupled to a solid support. This helps to reduce the background and can be used to separate or isolate the corresponding nucleic acid. The methods of attachment of DNA to a solid support have involved (1) non-specific physical adsorption of a single-stranded
15 DNA to nitrocellulose papers, and (2) covalent attachment via diazo coupling. Both methods are specific for single-stranded DNA. These covalent reactions are non-specific and several sites are coupled. These cause ineffective hybridization and loss
20 of perfect fidelity. Several points of attachment per chain reduces the flexibility of the DNA and reduces the rate of hybridization. Moreover, the lifetime of such an adduct is not very long. The DNA comes off easily and it is difficult to quantify the amount on the solid
25 support, without the use of radioactivity. The use of DNA probes for diagnostic purposes demands an effective method of tagging the DNA to a phase which can be separated easily from the rest of the nucleic acids.

30 U.S. Application Serial No. 511,063, filed on July 5, 1983, now pending in the names of Dattagupta, Rae and Crothers discloses various tests for nucleic acids, e.g., DNA of individuals being tested for sickle cell anemia. The test involves a soluble labelled probe and a probe fixed to a solid support. The probe can be
35 fixed to the support chemically as by a bifunctional reagent which at one end reacts with the support, e.g., a hydroxyl group of a cellulose molecule, and at the

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other end reacts with the DNA. This is quite satisfactory for many purposes but in some instances there may be too much bonding between the substrate and DNA, impairing the sensitivity of the DNA in the test.

5 It is accordingly an object of the present invention to provide a way of binding a nucleic acid to a solid substrate easily and without impairing the sensitivity of the DNA in the test.

10 These and other objects and advantages are realized in accordance with the present invention wherein there is provided a solid support capable of binding a nucleic acid thereto upon suitable irradiation, comprising (a) a solid substrate, (b) a photochemically reactive nucleic acid-binding ligand, and (c) a divalent radical
15 chemically linking the substrate and the nucleic acid-binding ligand.

The specific coupling reagents employed are functionalized, photochemically reactive nucleic acid-binding ligands, e.g., intercalator compounds such
20 as amino-substituted furocoumarins, e.g., amino-methyl-dimethyl-angelicin and amino-methyl-trimethyl-psoralen, and aminophenanthridium halides as well as closely related chemical derivatives thereof, and non-intercalator compounds such as netropsin, distamycin, Hoechst
25 33258 and bis-benzimidazole. Upon photoactivation these reagents will chemically link with nucleic acids. These reagents have a functionalized site other than the nucleic acid-reactive site and, by such other site, they are joined to a solid substrate, thereby in turn joining
30 the nucleic acid to such substrate with a minimum impairment of the nucleic acid function.

Apparently functionalized and photochemically reactive forms of a wide variety of intercalating agents can be used as the coupling reagent as exemplified in
35 the following table:

Intercalator Classes and
Representative Compounds

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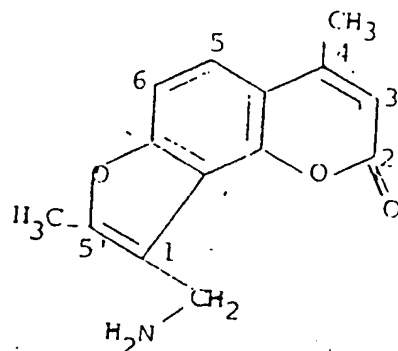
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15 Angelicin, more accurately 4'-aminomethyl-4,5'-
 dimethylangelicin, has the structural formula



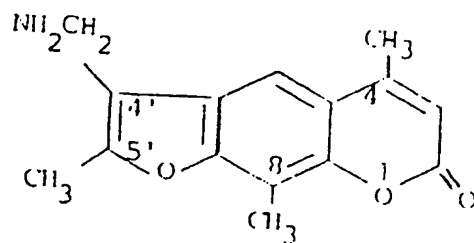
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(see Dall'Acquz et al, Photochemistry and Photobiology,
 Vol. 37, No. 4, pp. 373-379, 1983.)

Psoralen, more accurately 4'-aminomethyl-4,5',8-
 tri-methyl-psoralen (AMT), has the structural formula

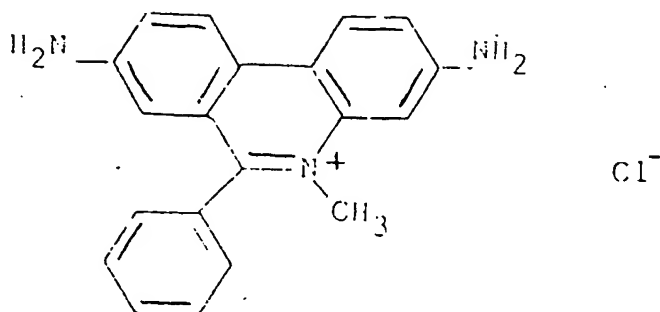
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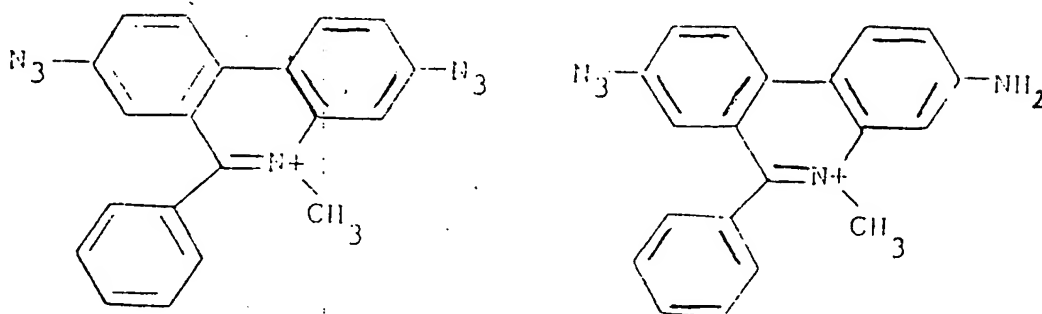
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(Cadet et al, Photochemistry and Photobiology, Vol. 37, No. 4, pp. 363-371, 1983.)

Methidium chloride, for example, has the formula



(see Graves et al, Biochemistry, 1981, Vol. 20 pp. 1887-1892.) Its mono- and di-azide analogues, shown below, are comparably reactive:



methidium monoazide

methidium diazide

as are the ethyl counterparts and the 4-(3-aminopropyl-N-carbamoyl) derivative of the phenyl side chain (methidium propylamine).

The solid substrate can be any solid which has reactive groups which could be carboxyl, amino or the like, but the preferred reactive groups are hydroxyl such as are found on cellulose. The cellulose may be unmodified as in cotton or paper or regenerated as in rayon or partially esterified as in cellulose acetate, cellulose propionate and especially cellulose nitrate,

or partially etherified as in methylcellulose and carboxymethylcellulose.

While the photochemically active intercalator reagent could be directly combined with the solid substrate, advantageously there is a mutual coupler which makes the connection. Suitable reagents include bifunctional compounds such as cyanogen bromide (CNBr), 1,4-butanediol diglycidyl ether, and the like. These are reacted with both the solid substrate and the photochemical reagent simultaneously or first with one and then with the other.

Thereafter, the product is further reacted with the nucleic acid photochemically. The reactions with the coupler and nucleic acid are substantially quantitative so the quantities of the reagents employed depend upon the desired ratio of nucleic acid to solid support. For most purposes, about 0.1 to 1000 mg and preferably about 1 to 100 mg of nucleic acid per gram of solid support will be suitable, although it may be higher or lower, depending upon the molecular weight of the nucleic acid, its sensitivity and the particular test in which it is to be used.

The reaction conditions in each step are generally known per se and any solvents and temperatures can be employed which permit the reactions to proceed without interference, e.g., from about -10 to 100°C., preferably about 10 to 50°C., and most preferably room temperature, employing inert organic solvents such as ether, carbon tetrachloride, THF, and the like.

The photochemically active reagents herein employed preferably react through amino groups. Identifying it as RNH_2 and the substrate with pendent OH groups as S, the stepwise reactions are as follows:

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The nucleic acid can be RNA or DNA or short (oligonucleotide) or long chain length, as desired, doubly or singly stranded.

5 Formation of monoadducts is desirable for hybridization experiments. In crosslinks, both DNA strands are covalently linked to psoralen chromophore and hence strand separation prior to hybridization is difficult. If the probe to be hybridized is linked to another non-specific piece of DNA, the non-specific part can be linked either via crosslink or monoadduct formation. In this case, irradiation can be done at 10 any wavelengths between 300-390 nm. Irradiation at 390 nm produces monoadduct; irradiation at 360-300nm produces both monoadduct and crosslinks.

15 If angelicin compounds are used, the product will predominantly be monoadduct irrespective of the wavelength of irradiation.

The invention will now be further described with reference to the accompanying examples wherein all 20 parts are by weight unless otherwise expressed.

Example:

1. Activation of the solid support and coupling of AMT.

25 The procedure described below has been followed for Sephadex G25 and cellulose, but any hydroxy-containing solid support can be activated by an identical procedure.

30 a) Activation with 1,4-butanediol-diglycidyl ether.

0.5-1 gm solid powder is swollen with water and washed, then 5-10 ml sodium hydroxide solution (0.5 M) is added. To this thick suspension, 1 ml 1,4-butanediol-diglycidyl ether is added. The 35 suspension is shaken overnight on a mechanical shaker and then washed with sodium hydroxide (0.5 M) solution and 1.0 ml 4'-aminomethyl-4,5',8-trimethyl-psoralen (2 mg/ml) in water is added, followed by enough 1 M sodium

hydroxide to have a thick suspension. The suspension is then stirred gently for 24 hours at room temperature and excess unreacted residues are quenched with lysine.

5 The solid is then washed with water followed by the desired aqueous buffer solution for DNA coupling.

b) For epoxidation of paper the identical procedure is followed with Whatman filter papers type 540, 1 and 541. The filter papers are taken on a watch glass or beaker cover (glass) and turned occasionally by hand. The rest of the procedure is the same as above.

c) Activation by cyanogen bromide and coupling of AMT. Typical example with cellulose:

15 0.5 gm cellulose is swollen in 5.0 ml distilled water for one hour. The swollen gel is washed thoroughly with distilled water. Then it is taken in an erlenmeyer flask, ice-cooled distilled water is added to the swollen cellulose and the pH is adjusted between 10.5 - 11 with 5M sodium hydroxide solution.

20 The flask with its contents is cooled in ice to avoid temperature rise above 15°C. 1 gm of solid cyanogen bromide is added to the cellulose and the solution is stirred for 30 minutes and pH maintained between 10.5 - 11 by NaOH. The suspension is washed with ice cold

25 distilled water, water is removed by centrifugation and 20 ml ice cold potassium phosphate buffer (10 mM; pH 8) is added. The activated cellulose is kept in brown bottles (in small aliquots) at - 20°C.

30 2 - 3 ml of swollen, activated gel is taken in a brown bottle and 0.7 ml AMT (2mg/ml) is added and the mixture is shaken gently in the cold room. Excess activated residues are quenched with lysine. The solid is washed with aqueous buffer for DNA binding.

35 d) For papers, similar procedures have been followed with Whatman cellulose filter papers type 540, 1 and 541 quantitative papers. Care should be taken to avoid tearing of the papers.

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e) Parallel experiments with ^3H labelled aminomethylpsoralen or angelicin are used to estimate labelling efficiency.

2. Coupling of phenanthridium compounds to a solid support and azide formation for photochemical coupling of DNA:

Activation of the solid supports is done by the method described above. As an example, methidium propylamine (R.P. Hertzberg and P.B. Dervan, JACS, 104, 313 (1982)) is coupled to the solid support, using identical buffer conditions as in 1. The isolated methidium containing solid support is then diazotized and azide derivative is made as follows. 1 gm cellulose or (2x5 cm²) of a sheet of activated paper containing methidium chloride is taken in 20 ml water, cooled in ice, 0.2 ml ice cold HCl is added; sodium azide (20 mg solid; 2x) is added. The vessel is cooled in ice and sodium nitrate solid (100 mg) is added. The reaction is allowed to proceed for 30 minutes, solid support is washed with the desired buffer. Coupling of DNA and hybridization are carried out the same way as described for aminomethyl-psoralen. Aminomethyl-dimethyl-angelicin can be similarly treated.

3. Photochemical coupling of DNA:

0.5 ml (0.2 - 0.3 gm gel + buffer) activated solid powder or 0.8 x 1 cm² activated paper is taken in a 1 cm path length spectrophotometer cuvette. Adenovirus DNA (partially labelled with ^3H) (concentration 25 µg/ml) in tris EDTA buffer (10 mM tris, 1mM EDTA, pH 7.5) is added to the cuvette and irradiation is done at a desired wavelength for 30 minutes to two hours depending on the future needs. For AMT, irradiation at 390 nm produces monoadduct whereas at 360 - 300 nm both monoadduct and crosslinks are formed. By altering the concentration and DNA sequence, crosslink to monoadduct formation can be modulated. After photoirradiation, the solid is washed and the radioactivity of the

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5a. The product of 4 is irradiated at 260 nm in otherwise the same manner as in 3, whereupon the DNA uncouples from the solid support, entering the solvent medium, viz. aqueous buffer. Then the liquid is assayed for ^3H .

It will be understood that the specification and examples are illustrative but not limitative of the present invention and that other embodiments within the spirit and scope of the invention will suggest themselves to those skilled in the art.

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CLAIMS:

1. A solid support capable of binding a nucleic acid thereto upon irradiation, comprising (a) a solid substrate, (b) a photochemically reactive nucleic acid-binding ligand, and (c) a divalent radical chemically linking the substrate and the nucleic acid-binding ligand.

2. A support according to claim 1, characterized in that the nucleic acid-binding ligand is an intercalator compound selected from acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines, and quinolines.

3. A support according to claim 1 or 2, characterized in that the substrate (a) in free state has free OH groups through which it is linked by the divalent radical (c).

4. A support according to any of claims 1 to 3, characterized in that the substrate (a) is cellulose or a cellulose ester.

5. A support according to any of claims 1 to 4, characterized in that the divalent radical (c) is derived from cyanogen bromide or from 1,4-butanediol-diglycidyl ether.

6. A support according to any of claims 1 to 5, characterized in that (b) is psoralen, angelicin, ethidium or derivatives thereof.

7. An immobilized nucleic acid probe comprising (a) a nucleic acid, (b) a nucleic acid-binding ligand photochemically linked to the nucleic acid, and (c) a

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solid substrate chemically linked through a divalent radical to the nucleic acid-binding ligand (b).

8. An immobilized probe according to claim 7,
characterized in that the nucleic acid-binding ligand
is an intercalator compound selected from acridine dyes,
phenanthridines, phenazines, furocoumarins, phenothiazines,
and quinolines.

9. An immobilized probe according to claim 7 or 8, characterized in that the substrate (c) in free state has free OH groups through which it is linked by the divalent radical.

10. An immobilized probe according to any of claims 7 to 9, characterized in that (b) is psoralen, angelicin, ethidium or derivatives thereof.

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EUROPEAN SEARCH REPORT

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A	CHEMICAL ABSTRACTS, vol. 88, no. 11, 13th March 1978, page 99, no. 69689r, Columbus, Ohio, US; L. KITTLER et al.: "Photochemically induced binding of furocoumarins with lambda phage DNA in situ", & STUD. BIOPHYS. 1977, 66(3), 237-41 * Abstract *	1,2	C 12 Q 1/68 // G 01 N 33/548 G 01 N 33/547
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Place of search THE HAGUE		Date of completion of the search 23-04-1986	Examiner OSBORNE H.H.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	



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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 23-04-1986	Examiner OSBORNE H.H.
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